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Variant *GDF9* mRNA is likely not the main cause of larger litter size in Iranian Lori-Bakhtyari, Shal, Ghezel, and Afshari sheep breeds

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Abstract. This study was carried out to screen the *GDF9* gene and evaluate the polymorphism effect on litter size of four Iranian sheep breeds using the PCR-RFLP and PCR-SSCP methods. First, sequencing of the *GDF9* gene in 16 twin-birth, 4 triplet-birth, and 2 infertile ewes showed that, in addition to G2, G3, G4, G5, and G6 mutations that have been previously reported in other breeds, a new G0 mutation, called C25T, exists in the *GDF9* sequence of 1 out of 22 ewes and causes L9F substitution in the signal peptide region. None of the triplet-birth or infertile ewes carried G1, G4, G7, FecGE, G8, or FecGT mutations. In the second experiment, a large dataset was used: 605 individuals including 496 ewes (145 Afshari, 54 Shal, 126 Ghezel, and 171 Lori-Bakhtyari sheep), and 109 rams (26 Afshari, 23 Shal, 10 Ghezel, and 50 Lori-Bakhtyari sheep. There were no sheep carrying the G7, G8, or Thoka mutations. Among all 109 rams that were used in this study, none of them were homozygous for the G1 mutation. Moreover, abundance of heterozygote rams (G1/G+) varied from 0.0 (Afshari) to 28.6 % (Lori-Bakhtyari and Ghezel). The highest and the lowest frequencies of the G4 mutation were 30.6 and 3.0 % in Shal and Afshari breeds, respectively. Moreover, G4 abundance varied from 0.0 to 42.3 %, from 3.0 to 26.9, and from 3.0 to 30.6 % in rams, ewes, and overall, respectively. There was a significant difference in the abundance of G1 and G4 mutations between breeds. However, neither the G1 nor the G4 mutation was associated with litter size in Afshari, Ghezel, Lori-Bakhtyari, or Shal sheep breeds. In conclusion, the results of this study showed that *GDF9* G1 and G4 mutations are not the reason for higher litter size in Iranian sheep. Moreover, the *GDF9* G0 and G6 mutations do not cause triplet births or infertility in Iranian ewes. Therefore, it is unlikely that variant *GDF9* mRNA induces larger litter size or infertility in Iranian ewes.

1 Introduction

In mammals, ovulation rate and fetus survival are decisive managerial attributes. It has been well-documented that single-nucleotide polymorphism (SNP) in a narrow assembly of genes, including growth differentiation factor 9 (*GDF9*), bone morphogenetic protein 15 (*BMP15*), bone morphogenetic protein receptor 1 (*BMPR1B*), and leptin, can increase ovulation rate, multiple-lamb births, and fecundity in sheep (Souza et al., 2001; Wilson et al., 2001; Hanrahan et al., 2004; Juengel et al., 2004, 2015). These genes belong to the transforming growth factor β (TGFβ) superfamily, which consists of 50 physiologically important macromolecules that regulate fertility and growth attributes, as well as cellular differentiation processes (Dong et al., 1996; Yan et al., 2001).

The importance of GDF9 protein on oocyte and follicular growth and function was defined by Dong et al. (1996) and McGrath et al. (1995), and afterward the ovine *GDF9* gene was mapped on chromosome 5 (Sadighi et al., 2002). Even though the *GDF9* gene is expressed in the oocyte of cumulus-oocyte complex (COC) (McGrath et al., 1995; Laitinen et al., 1998), its higher expression in the cellular layers around the antral follicles has recently been identified as key during the follicular phase of ewes (Foroughinia et al., 2017). As summarized in Table 1, following the first publication on discovering eight mutations, designated as G1 to G8, in the *GDF9* gene of Belclare and Cambridge breeds (Hanrahan et al., 2004), other mutations were discovered in Thoka (FecGT) (Nicol et al., 2009), Han (FecG-Han) (Chu et al., 2011), and various Spanish (FecGE) (Silva et al., 2011) sheep breeds. Among these mutations, G2, G3, G5 (Chu et al., 2011), and G-C (Nicol et al., 2009) are ineffective mutations without amino acid codon alteration. However, G1, G4, G6 (Chu et al., 2011), and FecGH (López-Ramírez et al., 2014) modify the GDF9 propeptide, while G7, G8 (Chu et al., 2011), G-D (Juengel et al., 2011), and FecGT (Chu et al., 2011) mutations cause variation in the mature peptide. Two of these SNPs, *GDF9* G8 and FecGT, show over-dominant inheritance for ovulation rate and twin birth but an infertility event at a homozygous mutant state (Hanrahan et al., 2004; Nicol et al., 2009). Moreover, the FecGE (Silva et al., 2011), G1 (Barzegari et al., 2010; Javanmard et al., 2011), G4 (Eghbalsaied et al., 2012, 2014), and G7 (Våge et al., 2013) mutations were reported as ovulation inducers without showing sterility. It has been proven that the origin of FecGH in Belclare and Cambridge sheep is the highly prolific Lleyn breed (Mullen et al., 2013). However, a large proportion of rams and high-fecundity ewes from the Lleyn breed and other highly prolific ewe breeds that had records of triplet births did not carry the known significant mutations (Mullen et al., 2013). This might indicate that other mutations in the detected major genes or other genes from the transforming growth family could affect the ovulation rate in ewes.

Mutation in the *GDF9* gene has been detected in Iranian sheep breeds, i.e. Zel (Ghaderi et al., 2010; Javanmard et al., 2011; Nassiry et al., 2006), Lori-Bakhtyari, Sangesari (Hafezian, 2011), Moghani (Barzegari et al., 2010), Ghezel (Akbarpour et al., 2008; Barzegari et al., 2010; Eghbalsaied et al., 2014), Shal (Ghaffari et al., 2009), Kurdi, Arabi (Ghaderi et al., 2010), Baluchi (Moradband et al., 2011), Afshari (Eghbalsaied et al., 2012), Mehraban (Zamani et al., 2015), and Lori (Zamani et al., 2015). However, all of these SNPs moderately modify the signal peptide or GDF9 propeptide. Conversely, neither the major known mutations that can change the mature peptide nor the ewe sterility that is the main side effect of ewe homozygosity for these major SNPs have been observed in Iranian ewes (Akbarpour et al., 2008; Eghbalsaied et al., 2012, 2014; Ghaffari et al., 2009; Moradband et al., 2011; Nejhad and Ahmadi, 2012). Even though the *GDF9* G1 mutation was not considered as an effective mutation for sheep prolificacy by Hanrahan et al. (2004), it was suggested as an effective source for inducing twin birth in Iranian Ghezel and Moghani breeds (Barzegari et al., 2010; Javanmard et al., 2011). However, further research on the Mehraban breed (Abdoli et al., 2013) could not prove the significant effect of the *GDF9* G1 SNP on Iranian sheep flocks. Also, the effect of other mutations, such as *GDF9* G4 (Eghbalsaied et al., 2012) and *GDF9* G6 (Khodabakhshzadeh et al., 2016), needs to be evaluated in highly fertile Iranian ewes. Therefor, evaluation of *GDF9* mutations in twin births of Iranian ewes remains to be explored in a large dataset of Iranian sheep breeds. The aim of this study was to screen and analyse *GDF9* polymorphism effects on twin births of Iranian sheep breeds, including Lori-Bakhtyari, Shal, Ghezel, and Afshari.

2 Material and methods

2.1 Experiment 1

Screening of the *GDF9* gene in a sample of twin-birth ewes

To search for possible mutations that are segregated in ewes with larger litter size sheep, a random sample of 16 ewes with twin births, 4 ewes with triplet births, and 2 ewes with infertility were selected from Shal, Ghezel, Afshari, and Lori-Bakhtyari breeds. The blood was immediately mixed with 50 mM EDTA, transported to the laboratory, and stored at −20 °C for further analysis. Genomic DNA was extracted from the whole blood using the standard phenol–chloroform method. The quantity and quality of extracted DNA was measured by spectrophotometer and agarose gel electrophoresis, respectively. Based on the *Ovis aries* breed Texel chromosome 5, Oar_v3.1, whole genome shotgun sequence on the National Center for Biotechnology Information (NCBI) website, three primer pairs were designed by Oligo6 software to cover the 5' UTR and complete sequence of exon 1, com-

Table 1. A summary of known mutations that have been detected in the *GDF9* gene of sheep species around the world.

Reference	Protein segment	Amino acid change	DNA base change	SNP
This paper	Signal peptide	L9F	C25T	G0
Hanrahan et al. (2004)	Propeptide	R87H	G260A	G1
Hanrahan et al. (2004)	Propeptide	Unchanged V	C471T	G2
Hanrahan et al. (2004)	Propeptide	Unchanged L	G477A	G3
Hanrahan et al. (2004)	Propeptide	K241E	A721G	G4
Chu et al. (2011)	Propeptide	Q243H	G729T	FecG-Han
Nicol et al. (2009)	Propeptide	Unchanged R	G750A	G-C
Juengel et al. (2011)	Mature peptide	R286S	G858T	G-D
Hanrahan et al. (2004)	Mature peptide	Unchanged E	A978G	G5
Hanrahan et al. (2004)	Mature peptide	V332I	G994A	G6
Silva et al. (2011)	Mature peptide	F345C	T1034G	FecGE
Hanrahan et al. (2004)	Mature peptide	V371M	G1111A	G7
Hanrahan et al. (2004)	Mature peptide	S395F	C1184T	G8 (FecGH)
Nicol et al. (2009)	Mature peptide	S427R	A1279C	FecGT

Table 2. Distribution of collected samples from ewes and rams belonging to Iranian sheep breeds.

Breed	Ewe				Ram	Location	Total number
	Triplets	Twins	Single	Infertile			
Shal	3	30	19	2	23	Pir Yusefiyan (Bu'in Zahra)	77
Ghezel	–	10	30	–	10	Miandoab Research Center	136
	–	52	34	–	–	Semmeneh Rud (Boukan)	
Afshari	–	70	30	–	6	Khatoon Abad (Isfahan)	171
			45		20	Isfahan	
Lori-Bakhtiyari	1	110	60	–	50	Shahrekord, Lordegan, Farsan	221
Total number	4	272	218	2	109	Iran	605

plete sequence of exon 2, a part of exon 2, and the 3' UTR of the ovine *GDF9* gene (Table 2). Polymerase chain reactions (PCR) were carried out in 25 µL volume, included 1X Buffer, 250 mM dNTP, 5 mM $MgCl_2$, 5 µM primers, 50–100 ng template DNA, and 1 IU Taq DNA Polymerase (SinaClon, Iran). Except for the annealing temperature, PCR conditions were similar for all reactions, with initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 s, annealing at 55–60 °C for 30 s, and extension at 72 °C for 30 s, and finished by a final extension at 72 °C for 4 min. The PCR amplicons of the 16 ewes were sent for sequencing (Bio Basic Inc., Canada) and aligned with the *Ovis aries GDF9* mRNA; sequence ID is gb|AF078545.2|AF078545.

2.2 Experiment 2

2.2.1 DNA samples and PCR reaction

In this experiment, the detected mutations in Experiment 1 were assessed using twin births of four main breeds of Iranian sheep. Five millilitres of a blood sample was collected from the jugular vein of 605 sheep, including 77, 136, 171, and 221 Shal, Ghezel, Afshari, and Lori-Bakhtyari breeds, respectively (Table 3). Among these animals, four triplet-birth and two infertile ewes were also included. Five primer pairs were used for detection of G0, G1, G4, G6, and G8 mutations in *GDF9* gene (Table 2) (Polley et al., 2010). The PCR conditions were similar to Experiment 1.

2.2.2 Restriction fragment length polymorphism (RFLP)

For detection of G1 and G8 mutations, 1 µg of the PCR product was digested with *Hha*I and *Dde*I restriction enzyme, respectively (Takara Bio Inc., Japan), at 37 °C for 1 h. Then the digested solution was loaded on a 1.5 % agarose gel containing GelRed (Biotium, USA) and screened using gel documentation (Uvitech, UK) merchandiser.

2.2.3 Single-strand conformation polymorphism (SSCP)

Detection of the G0, G4, G6, and G8 mutations as well as the G0 mutation that was newly detected in Experiment 1, was carried out using the SSCP procedure (Orita et al., 1989). In summary, 5 µL of the PCR product was transferred to an Ep-

Table 3. Primer pairs for mutation detection in the *GDF9* gene of Iranian sheep based on NCBI accession reference AF078545.

Coverage region	Primer sequence $5' \rightarrow 3'$	Amplicon length (bp)	Tm (C)	Reference
G0	AGAACTGCAATTCCACTCAAGATT GCCTTCCTCATGGCCAAATG	161	59	Current paper
G1	TCTTCTTCCCTCCACCCATTAACCAATC GCCTGGCTCTGTTTTCCTATTAGCCTTG	396	61	Polley et al. (2010)
G4	CCTGCTGGGTTAGAAGGGTT TTCCCCACGTTTGTTGCTTTC	195	60	Current paper
G6	GATGCTAACCTCCAGCAGCA TGCCCTCATGGGTTGATGTAG	91	60	Current paper
G8	GGATTGTGGCCCCACACAAATACAACCC CATCAGGCTCGATGGCCAAAACACTCAA	198	55	Polley et al. (2009)
$5'$ UTR + exon 1	GAATGAATAGGGTGTTGTCAGC TTAAGGCCATTAAATCTCTTCTAGC	809	59	Current paper
Exon 2	CCCCACCAAAGCTATTCTGA CACCCTCAGCAGCTTCTTCT	757	61	Current paper
$3'$ UTR	TTACATGTGCGGAAGACCAG AACATTTGGCCATGAGGAAG	859	60	Current paper

pendorf tube and mixed with 7.0 µL of a gel loading solution that contained 99 % formamide, 0.05 % bromophenol blue, 0.05 % xylene cyanol, and 20 mM EDTA (pH = 8.0). The mixture was incubated and denatured at 96 °C for 10 min, chilled on ice for 5 min, and loaded onto 12 % neutral polyacrylamide gels. Electrophoresis was performed at 130 volt for 18 h at 10–12 °C. Afterward, the single-strand DNA was made visible using silver staining (Bassam et al., 1991).

3 Statistical analysis

The following generalized linear model (GLM) was used for evaluation of the detected polymorphisms in ewe twin births.

$$\text{Twinbirth} = \text{mean} + \text{breed} + \text{G1} + \text{G4} + \text{E}, \quad (1)$$

where mean, breed, G1, G4, and E were the average of twin births in the whole population. Breed and genotype of ewes for the G1 and G4 mutations were fixed effects, and unknown residual effects were random effect. The statistical analysis was carried out using SAS 9.2 software. Mean of litter size was compared between genotype categories using the least significant difference test (p value < 0.05). Genetic analysis was carried out using the Popgene 1.32 software (Francis and Yang, 2000) and genotypic and allelic frequencies were compared using the Chi-square test (p value < 0.05).

4 Results

4.1 Detection of mutations converting L9F (G0) and V332I (G6)

In the first part of this study, the pair-wise alignment results of the sequenced fragments and the *Ovis aries GDF9* mRNA were used to determine possible mutations in the surveyed samples from twin-birth, triplet-birth, and infertile ewes. As depicted in Fig. 1, results showed that along with the G1 mutation, five previously reported mutations, namely the *GDF9* G2, G3, G4, G5, and G6 (Hanrahan et al., 2004), are present in highly prolific Iranian ewes with 3.1 (1 out of 16), 3.1 (1 out of 16), 7.1 (3 out of 21), 30.0 (6 out of 20), and 30.0 % (6 out of 20) frequency, respectively. Furthermore, we detected a new mutation, designated as G0, in one out of the 22 sequenced samples. This mutation exists in exon 1 of the *GDF9* gene, causing C25T to shift and subsequently L9F to convert into the amino acid polypeptide. This amino acid change occurs in signal peptide of GDF9 protein (Senta et al., 2009) and is completely conserved among sheep [NP_001136360.2], goats [NP_001272637.1], cattle [NP_777106.1], dogs [NP_001161485.1], wild boar [NP_001001909.1], humans [NP_001275754.1], and mice [NP_032136.2] (Fig. 2). The *GDF9* G6 mutation was the only detected mutation that affected the active polypeptide sequence. We did not detect G7, G8, FecGT, and FecGE mutations in the evaluated samples.

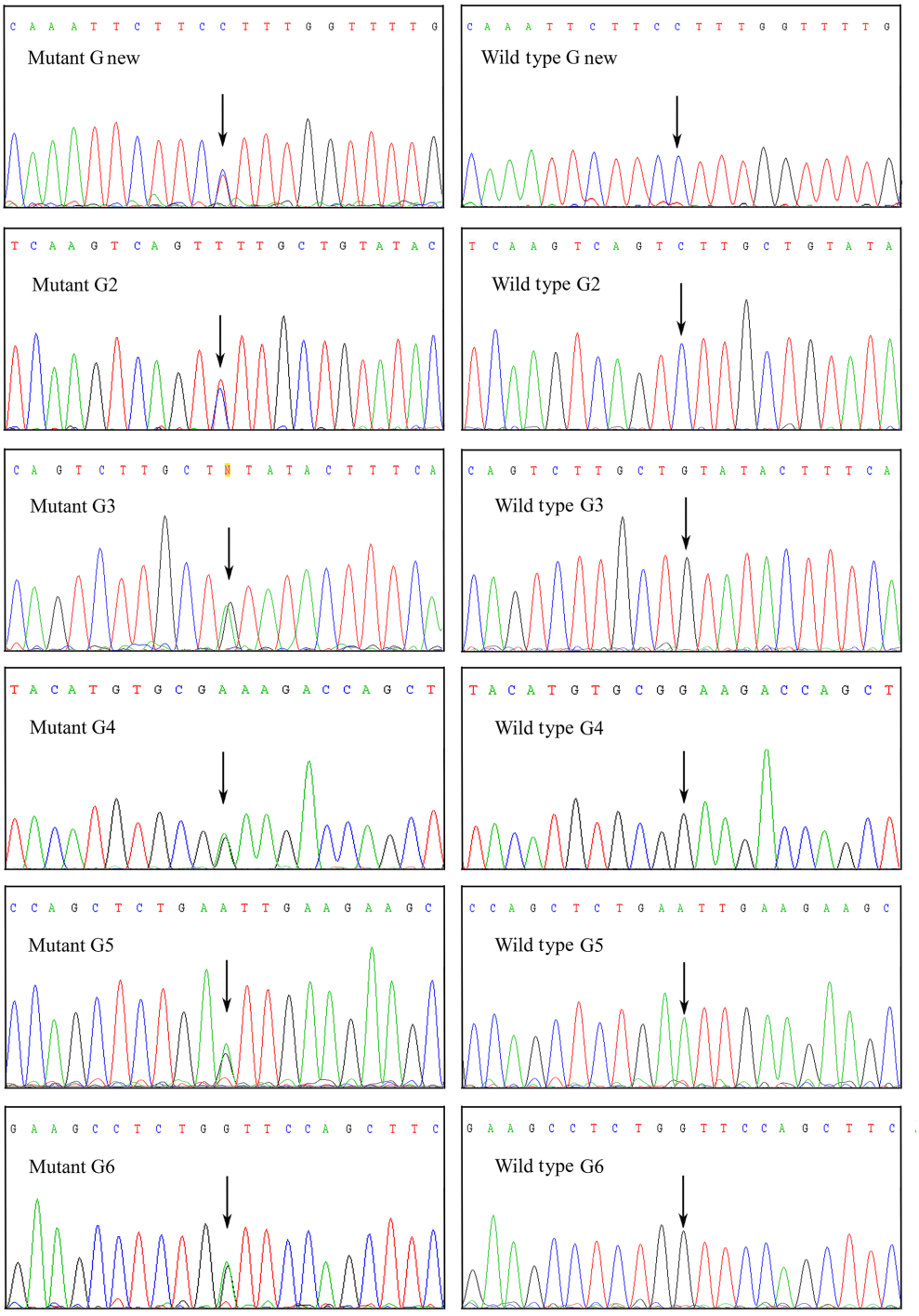

Figure 1. Graphical representation of detected mutations in *GDF9* DNA of Iranian twin-birth ewes compared to sequence ID gb|AF078545.2| AF078545. G0 (C25T) is the newly detected mutation; G2–G6 were previously defined by Hanrahan et al. (2004) .

4.2 G1 and G4 SNPs were unimportant for twin births in Iranian ewes

In this study, we collected a large sample size of sheep from four main breeds in Iran, including Afshari, Ghezel, Lori-Bakhtyari, and Shal. Four primer pairs were used to amplify DNA sequences that cover the G0, G1, G4, and G6 mutations. We used a SSCP approach for discriminating the possible alleles in the studied population. However, amplicons that contain G0 or G6 mutations were not distinguishable using the SSCP method, although we included the animals that

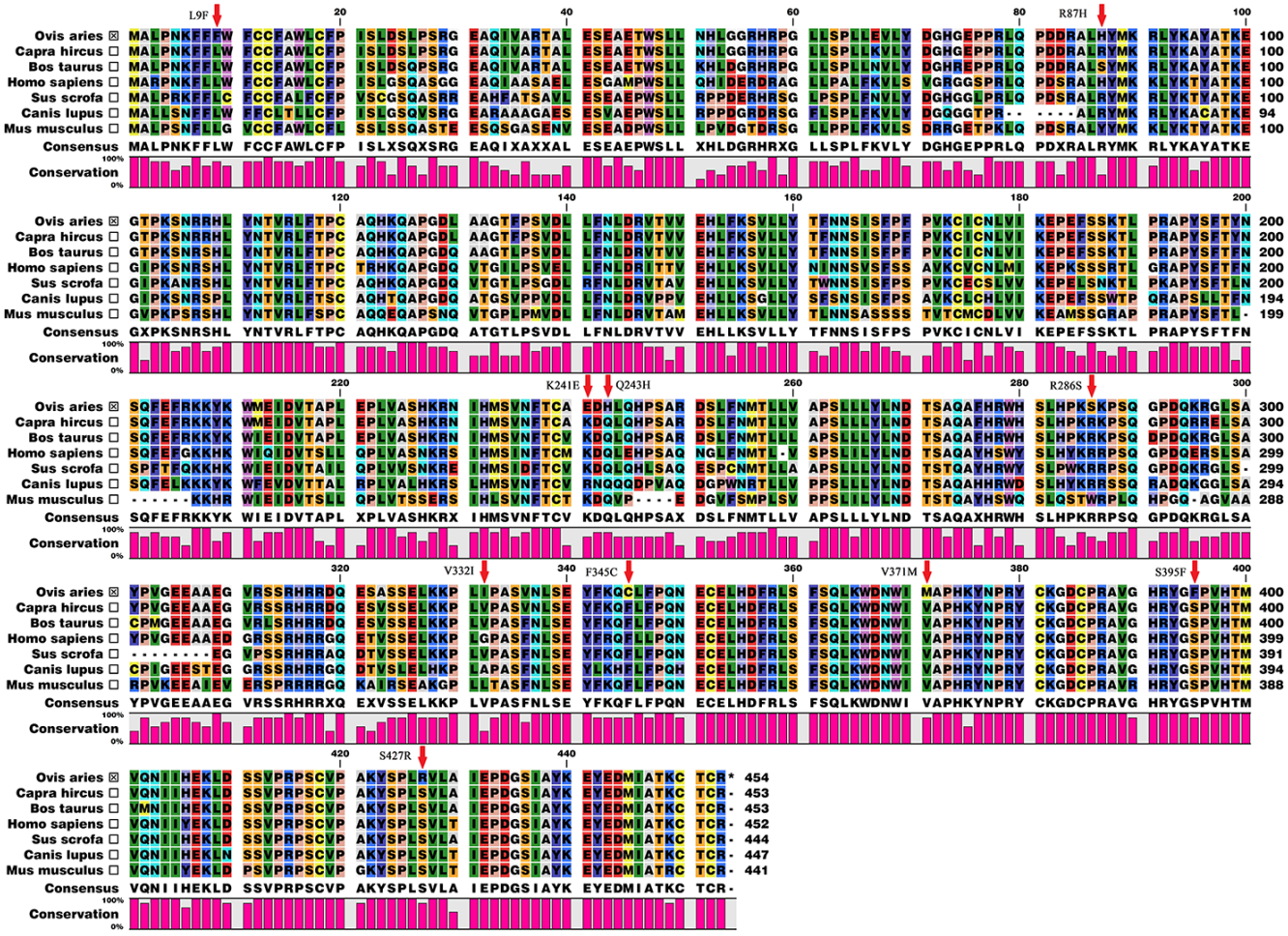

Figure 2. Multiple alignment of GDF9 propeptide sequence among sheep [NP_001136360.2], goats [NP_001272637.1], cattle [NP_777106.1], dogs [NP_001161485.1], wild boar [NP_001001909.1], humans [NP_001275754.1], and mice [NP_032136.2]. The amino acid changes due to the detected *GDF9* mutations in sheep are illustrated by arrows.

were previously verified as heterozygous genotypes for these mutations.

Results of this study for genotypic and allelic frequencies for the G1 mutation (G260A) in Iranian sheep breeds are presented in Table 4. Among all 109 rams that were used in this study, none of them were homozygous for the G1 mutation. Moreover, abundance of heterozygote rams (G1/G+) varied from 0.0 (Afshari) to 28.6 % (Lori-Bakhtyari and Ghezel). Analysis of pooled data from ewes and rams indicated that there was a high difference in G1 allelic frequency as well as genotypic distribution among the four mentioned breeds (p value = 0.046). Although this allele was not detected in the Lori-Bakhtyari breed, its abundance was 14.0 % in the Ghezel breed. Our results based on the large dataset comprised of Afshari, Ghezel, Lori-Bakhtyari, and Shal breeds could not detect any significant effect of the G1 mutation on the litter size of ewes (Fig. 3) (p value = 0.991). In addition, neither the two infertile ewes nor the four triplet-birth ewes carried the G1 mutation.

The highest and the lowest frequencies of the G4 mutation were detected in the Shal and Afshari breeds, respectively (Table 5). G4 mutation frequency varied from 0.0 to 42.3 %, from 3.0 to 26.9, and from 3.0 to 30.6 % in rams, ewes, and overall, respectively. There was a significant difference in the abundance of the G4 mutation between breeds (p value = 0.003). However, no homozygote G4/G4 was observed in ewes and rams of the Afshari breed. Moreover, using this large dataset in the current study showed that the G4 mutation did not have a significant effect on ewe twin births (Fig. 4) (p value = 0.864).

5 Discussion

A high number of mutations were reported by Hanrahan et al. (2004) in Belclare and Cambridge sheep. The *GDF9* G1 mutation was previously detected in the Iranian Ghezel, Moghani, and Afshari breeds (Abdoli et al., 2016; Eghbalsaied et al., 2014; Noshahr and Rafat, 2014). Additionally,

Table 4. Allelic and genotypic frequencies of the *GDF9* G1 mutation (G260A) in Iranian Shal, Ghezel, Afshari, and Lori-Bakhtyari sheep breeds.

Breed	Sex	Genotype frequency (%)			Allelic frequency (%)	
		G+/G+	G+/G1	G1/G1	G+	G1
Shal	Male	93.7	6.3	0.0	96.9	3.1
	Female	81.8	9.1	9.1	86.4	13.7
	Overall	85.7	8.2	6.1	89.8	10.2
Ghezel	Male	71.4	28.6	0.0	85.7	14.3
	Female	74.0	24.0	2.0	86.0	14.0
	Overall	73.7	24.6	1.7	86.0	14.0
Afshari	Male	100.0	0.0	0.0	100.0	0.0
	Female	81.4	14.0	4.6	88.4	11.6
	Overall	85.4	11.0	3.6	90.9	9.1
Lori-Bakhtyari	Male	71.4	28.6	0.0	85.7	14.3
	Female	89.3	10.7	0.0	94.7	5.4
	Overall	88.2	11.8	0.0	94.1	5.9
Total		83.9	13.8	2.3	90.8	9.2

Table 5. Allelic and genotypic frequencies of the *GDF9* G4 mutation (G721A) in Iranian Shal, Ghezel, Afshari, and Lori-Bakhtiari sheep breeds.

Breed	Sex	Genotype frequency (%)			Allelic frequency (%)	
		G+/G+	G+/G4	G4/G4	G+	G4
Shal	Male	30.8	53.8	15.4	57.7	42.3
	Female	51.2	43.9	4.9	73.2	26.9
	Overall	46.3	46.3	7.4	69.5	30.6
Ghezel	Male	75.0	12.5	12.5	81.3	18.8
	Female	62.5	30.0	7.5	77.5	22.5
	Overall	64.6	27.1	8.3	78.2	21.9
Afshari	Male	0.0	0.0	0.0	0.0	0.0
	Female	94.1	5.9	0.0	97.1	3.0
	Overall	94.1	5.9	0.0	97.1	3.0
Lori-Bakhtyari	Male	66.6	16.7	16.7	75.0	25.1
	Female	61.4	28.4	10.2	75.6	24.4
	Overall	61.7	27.6	10.7	75.5	24.5
Total		63.5	28.7	7.8	77.9	22.2

the presence of *GDF9* G2, G3, and G4 SNPs was confirmed in the Iranian Afshari breed (Eghbalsaied et al., 2012). Moreover, existence of G5 and G6 mutations in the *GDF9* gene of Iranian ewes has also been recently reported (Khodabakhshzadeh et al., 2016). The *GDF9* G1 and G4 mutations convert arginine to histidine and glutamic acid to lysine in the pre-peptide but not the matured polypeptide. The G6 mutation converts valine to isoleucine in the active GDF9 protein. Both valine and isoleucine are classified into hydrophobic side-chain amino acids. It should be noteworthy that all significant mutations in the ovine *GDF9* gene, including G7, G8, FecGE, and FecGT, occur in a completely conserved region of the protein. Results of a recent publication indicated that unlike the suggestion by Hanrahan et al. (2004), the G7 missense mutation that causes valine to convert to methionine (both have a hydrophobic side-chain amino acid) does affect the twin birth rate in Norwegian white sheep (Våge et al., 2013). The existence of the G0 mutation, C25T, has recently been reported by the Ensembl website using the next generation sequencing data from Iranian (IROV) and Moroccan *Ovis aries* sheep (MOOV) with 2.5 and 0.6 % frequency, respectively (rs605683468). In agreement with this only sequencing report, the frequency of this mutation was 2.3 % with no homozygote genotype in the sequenced samples. We need to consider the point that only 1 out of 16 twin-birth ewes carried this mutation, while 94 % of twin-birth ewes, all four triplet-birth ewes, and two infertile ewes did not carry this mutation. Therefore, the C25T or G0 mutation is not likely the reason for twin births in Iranian ewes, although valine is conserved among sheep, goats, cattle and wild boar.

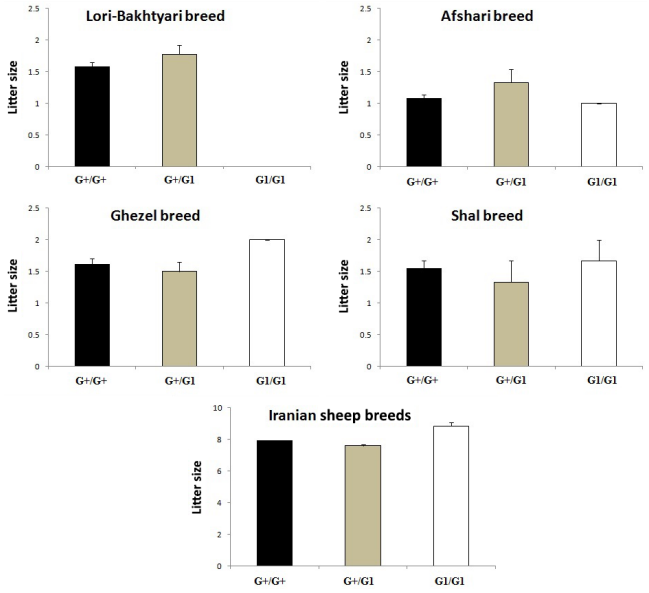

Figure 3. Litter size of Iranian ewes carrying the G1 mutation in the *GDF9* gene.

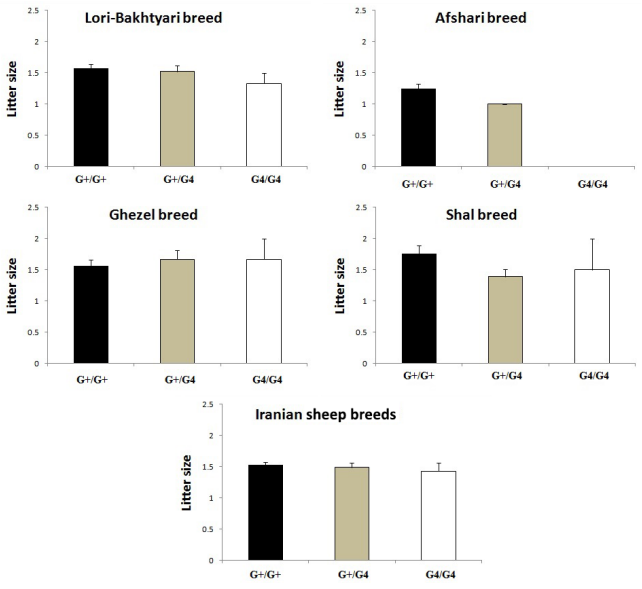

Figure 4. Litter size of Iranian ewes carrying the G4 mutation in the *GDF9* gene.

However, the G6 mutation was observed in 6 out of 14 sequenced samples from twin-birth records, all in a heterozygote state. However, neither the ewes with triplet births nor the infertile ewes carried this mutation. Although the SSCP technique could not differentiate the mutation in this study, we cannot rule out the possible partial effect of this mutation in litter size of Iranian ewes. Moreover, the G1 mutation alters a non-conserved region, even in ruminants, and the G4 mutation also supports conservancy rather than decreasing it. Moreover, both mutations occur in the pre-peptide region. Thus, these mutations might be less likely to be effective in altering *GDF9* activity. In the sequenced sample, we could not detect any homozygous genotype for these mutations, although, for example, the moderate frequency of G6 mutations indicated that we could expect around 4 % homozygosity for *GDF9* G6. This may be due to the fact that such mutations are segregated at very low frequency and thus they are highly affected by the sampling method. Therefore, using a larger sample size will be required for evaluation of the allelic and genotypic frequencies as well as the corresponding effect of these SNPs. Similarly, further mutations are expected to be detected by increasing the number of highly fertile ewes.

In the second phase of this study, we collected a large sample size of Iranian sheep. We tried to genotype the animals for G0, G1, G4, and G6 mutations. However, the SSCP approach could not detect either G0 or G6 mutations. This could be due to insensitivity of the SSCP procedure in detecting these mutations. There are several parameters such as type of filtering matrix, type of additive, wall coating, temperature, and voltage that can significantly alter the SSCP banding pattern (for review see Sinville and Soper, 2007). Insensitivity or low sensitivity of SSCP compared to other SNP detection technologies was also reported (Low et al., 2000). Although we used a standard range of parameters for SSCP optimization (Eghbalsaied et al., 2016; Khodabakhshzadeh et al., 2016), implementing this technology was not helpful for distinguishing possible mutations in these amplicons.

Using the RFLP and SSCP methods, the G1 and G4 mutations were distinguished, respectively. A considerable difference was observed between genotypic frequencies in different breeds so that this allele was not detected in the Lori-Bakhtyari breed, while it was present in 14.0 % of the Ghezel breed. Also, none of the sires in this study were homozygous for the G1 mutation. In the literature, the frequency of the G1 mutation in Iranian breeds has been reported as follows: 8.7–15.7 % in the Ghezel breed (Barzegari et al., 2010; Eghbalsaied et al., 2014), 2.7–24.0 % in the Afshari breed (Eghbalsaied et al., 2014; Javanmard et al., 2011), 0.0 % in the Shal breed (Eghbalsaied et al., 2014), 15.7 % in the Moghani breed (Barzegari et al., 2010), 18.0–23.0 % in the Baluchi breed (Javanmard et al., 2011; Moradband et al., 2011), 19.8 % in the Sangsari breed (Hafezian, 2011), 22.5 % in the Makui breed (Javanmard et al., 2011), and 18.0–40.6 % in the Mehraban breed (Abdoli et al., 2013; Javanmard et al., 2011). These genetic polymorphisms in Iranian sheep corresponded to a 24.0 % frequency in the Chios and Karagouniko breeds in Greece (Liandris et al., 2012), and 5.0–20.0 % in the Sal'skaya and Romanov breeds in Russia (Kolosov Yu et al., 2015).

The importance of the G1 mutation on ewe prolificacy is controversial in the literature. Although it has been suggested as an important mutation that increases twin births in the

Ghezel and Moghani breeds in Iran (Barzegari et al., 2010) as well as the Chios and Karagouniko breeds in Greece (Liandris et al., 2012), an adverse effect of this mutation on litter size was reported in Iranian Baluchi sheep (Moradband et al., 2011). Interestingly, an over-dominant effect of this gene was also reported for litter size of Iranian Mehraban, Afshari, Baluchi, and Makui breeds (Javanmard et al., 2011), while no important effect of the G1 mutation on litter size was reported in Mehraban ewes (Abdoli et al., 2013). Differences in the sampling size and the power of the SSCP vs. RFLP techniques may be the reason for these large discrepancies in the literature. Our results with a large sample size showed that the distribution of genotypic frequencies among single-birth and twin-birth ewes was not different in all the studied breeds. This clearly showed that the G1 mutation is not responsible for infertility or multiple-lamb births in Iranian ewes. It was suggested that G1 mutation (G260A) causes R87H changes in the amino acid chain, leading to replaced arginine instead of histidine in the pre-peptide region of the GDF9 protein and therefore it has no effect on ewe litter size (Hanrahan et al., 2004). The results of our study support the hypothesis of Hanrahan et al. (2004) and the findings of Abdoli et al. (2013) for Iranian sheep. In addition, lack of homozygous mutant rams in all four breeds in the current study indicated that this mutation does not favour twin births in Iranian ewes under natural or artificial selection.

The other mutation that was screened for in the current study on Iranian sheep breeds was (G721A) G4 (Hanrahan et al., 2004). The G4 mutation frequency varied significantly between breeds, so that it was estimated at 3.0 % in the Shal breed and 30.6 % in the Afshari breed. It is evident that the G4 mutation is present in the Iranian Afshari, Shal, and Sangsari breeds. The frequency of this mutation was estimated at 7.9 % in the Afshari breed (Eghbalsaied et al., 2014); 6.25 % in Shal (Eghbalsaied et al., 2014); 10.0 % in the Ghezel breed (Eghbalsaied et al., 2014); 19.0 % in Greek sheep breeds (Liandris et al., 2012); 44.8 and 3.8 % in the Belclare and Cambridge breeds of Ireland, respectively (Hanrahan et al., 2004); and 95.0 and 80.0 % in the Sal'skaya and Romanov sheep breeds of Russia (Kolosov Yu et al., 2015).

The *GDF9* G4 mutation was not significantly important for ewe twin births. The effect of the G4 mutation on twin births in Greek ewes was also not significant (Liandris et al., 2012). There are limited publications that address the frequency and the importance of the G4 mutation. There was one ewe, which was homozygous for G4 mutation, with a very high number of antral follicles (Eghbalsaied et al., 2012). The G4 mutation (G721A) causes glutamine–241–lysine conversion, which takes place in the GDF9 pre-peptide and is unlikely to affect *GDF9* functional activity (Hanrahan et al., 2004). More importantly, none of the four ewes with triplet births or the two infertile ewes carried the G1 or G4 mutations.

Screening the *GDF9*, *BMP15*, and *BMPR1B* genes in Davisdale sheep indicated that several mutations in *GDF9* and *BMP15* segregate in these breeds. However, none of these mutations were responsible for higher ovulation rate and litter size in these breeds (Juengel et al., 2011). Instead, mutation in the leptin receptor gene was significantly associated with delayed onset of puberty and a decrease in ovulation rate and litter size in these breeds (Haldar et al., 2014). Therefore, it is possible that mutations in other genes, such as *BMP15*, *BMPR1B* (Eghbalsaied et al., 2016), leptin, or leptin receptor, are responsible for higher antral follicle count, ovulation rate, and litter size in Iranian sheep breeds. A recent study showed that in addition to the bone morphogenetic protein signaling pathway, expression of genes involved in estrogen and AMPK synthesis can be important factors in antral follicle count in ewes (Foroughinia et al., 2017). Therefore, more comprehensive studies are required to determine effective genetic mechanism controlling ewe twin births in Iranian sheep breeds.

6 Conclusion

In this study, a new mutation was detected in the early pre-peptide region of the *GDF9* gene. In addition, the G1 and G4 mutations were highly variable among different breeds so that they were not observed in homozygote mutants in rams of some breeds. Moreover, neither the G1 nor the G4 mutation had an effect on ewe litter size. This clearly indicated that the selected rams in these flocks were neither naturally nor artificially selected for these mutations. In addition, the G8 mutation was not observed in these sheep breeds. None of triplet-birth or infertile ewes carried the G0, G1, G4, G6, G8, and Thoka SNPs. In conclusion, the results of this study suggest that the *GDF9* G1 and G4 mutations are not the reason for higher litter size or fecundity in Iranian sheep. Moreover, neither *GDF9* G0 nor G6 mutations cause triplet births or infertility in Iranian ewes. Therefore, it is unlikely that variant *GDF9* SNPs, which currently segregate in Iranian ewes, induce larger litter size or infertility.

Data availability. The data (sequencing data and SSCP results) used in this article can be found in the Supplement.

The Supplement related to this article is available online at doi:10.5194/aab-60-119-2017-supplement.

Competing interests. The authors declare that they have no conflict of interest.

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